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14. ABSTRACT:

This research project investigates the role of the IκB kinase (IKK) and NF-κB in the development of prostate cancer (CaP), and examines the possibility that IKK inhibitors can be used in CaP treatment. To reach this goal we are employing two mouse models in which either the IKKβ or the IKKα subunit of IKK are deleted or inhibited in prostate epithelial cells. We found that neither IKKβ nor IKKα are required for normal prostate development, however IKKα may play an important role in the development of advanced CaP. The study on the role of IKKβ in prostate carcinogenesis in animal models is ongoing. We also found that IKK/NF-κB activities were increased during the evolution of androgen-independent CaP, a response that could be mediated by some of androgen-regulated genes, such as TMEFF2. Furthermore, we found that a prototypical IKK inhibitor IT-3 can suppress the proliferation of human CaP cells. Although a more thorough examination of the role of IKK/NF-κB in CaP development and progression is currently underway, our results obtained during last year suggest and important role for both IKKα and IKKβ in development and progression of CaP. Therefore inhibition of either protein kinase or both would be an effective and attractive option of the treatment of CaP.

15. SUBJECT TERMS
IκB kinase (IKK), NF-κB, prostate cancer, inhibitors, carcinogenesis, treatment

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Introduction

There are two NF-κB activation pathways. The first pathway, the classical NF-κB activation pathway, is normally triggered in response to microbial and viral infections and exposure to proinflammatory cytokines which activate the three subunit IKK complex leading to phosphorylation-induced degradation of IκBs. This pathway depends mainly on the IKKβ catalytic subunit. The other pathway, the alternative pathway, leads to activation of p52:RelB dimers by inducing processing of the NF-κB2/p100 precursor protein that binds RelB in the cytoplasm. This pathway is triggered by certain members of the tumor necrosis factor (TNF) cytokine family through selective activation of IKKα homodimers by the upstream kinase NIK.

There is considerable evidence that the two IKK/NF-κB signaling pathways are involved in carcinogenesis, cancer progression, metastasis and drug resistance. Although certain viral proteins, cancer-associated chromosomal translocations, and mutations can lead to constitutive activation of NF-κB in cancer progenitor cells, the most common mechanism leading to NF-κB activation during tumorigenesis depends on autocrine and paracrine production of proinflammatory cytokines. Persistent activation of NF-κB can lead to increased production of tumor growth factors by components of the tumor stroma as well as to upregulation of anti-apoptotic genes within the cancer cell itself. This process was recently demonstrated to occur during two different mouse models of inflammation-associated cancer leading to development of colorectal cancer and hepatocellular carcinoma. We also provided evidence for a role of NF-κB in inflammation-driven metastatic growth. In that model, as well, IKK-driven NF-κB activation is responsible for production of growth and survival factors by stromal components (macrophages) and upregulation of anti-apoptotic genes within the cancer cell.

Prostate cancer (CaP) is one of the most common cancers in men and the second leading cause of cancer-related deaths among men in the United States. It was shown that NF-κB transcription factors can directly interact with several members of the nuclear receptor family including androgen receptor (AR) itself. Thus, NF-κB may function as a co-activator for AR causing it to be active independently of androgen binding. In this case, prostate epithelial cells with high NF-κB activity are rendered resistant to androgen withdrawal. However, NF-κB can also trans-repress ligand-bound AR or repress the AR gene itself. Most importantly, NF-κB activity itself is repressed by androgen treatment via AR-mediated trans-repression or other mechanisms. In this case, NF-κB activity may increase in response to androgen withdrawal. Once activated, NF-κB can stimulate production of various cytokines by prostate epithelial cells, CaP and stromal components. Specifically, IL-6, a well-known autocrine and paracrine growth factor for AI CaP cells, is encoded by a typical NF-κB target gene. The dependence of IL-6 expression on NF-κB was demonstrated in the prostate epithelium. Importantly, the human AD CaP cell line, LNCaP, was shown to assume a neuroendocrine (NE) phenotype in response to IL-6 exposure. Although NE cancers of the prostate are rare, foci with NE-like features can be observed in nearly all prostate adenocarcinomas and extensive NE differentiation is generally considered to be of poor prognostic value. Indeed, NE differentiation appears more frequently in hormone-refractory cancer. Inhibition of IL-6 activity induces the regression of AI human CaP xenografts in mice. Thus, NF-κB inhibition should result in a similar effect by inhibiting IL-6 expression. Studies performed on various CaP cell lines reveal that AI cells often display constitutive NF-κB activity. Furthermore, constitutively activated NF-κB in three different human CaP cell lines was linked to overexpression of IKK subunits, and inhibition of NF-κB activity in these cells through expression of a non-degradable super-repressor mutant of IκBα resulted in either spontaneous apoptosis or increased sensitivity to TNFα.

SPECIFIC AIMS

- Construct mice with a conditionally activated Ikkβ allele and use them to determine wether constitutive NF-κB activation in prostrate epithelial cells promotes prostate cancer.

- Construct mice with a specific deletion of Ikkβ in prostate epithelial cells and examine whether this deletion, as well as the inactivation of IKKα, inhibits prostate carcinogenesis in the TRAMP model.

- Use various strategies to inhibit IKK activity and test them for their ability to inhibit proliferation and induce apoptosis in prostate cancer cell lines of human origin.

Key Research Accomplishments

1. Examine whether inactivation of IKKα inhibits prostate carcinogenesis in the TRAMP model.

To investigate the role of the IKKα subunit, which may offer a more attractive target for drug development than IKKβ as it is not required for innate immune responses, TRAMP mice were crossed with *Ikkα*$^{AA/AA}$ mice which express a form of IKKα that can not be activated because the two serines in its activation loop, which are phosphorylated by the upstream activating kinase NIK, were replaced with alanines. The resultant *Ikkα*$^{AA/AA}$*/TRAMP* mice were monitored for tumor development and found to exhibit fewer metastases (including metastasis to lymph nodes and other organs) and survive considerably longer than *WT/TRAMP* mice (Fig. 1 and Table 1). The CaP in *Ikkα*$^{AA/AA}$*/TRAMP* mice exhibit reduced cell proliferation (Fig. 2). However, there are no differences in size and weight of the prostate glands between 12-week-old *Ikkα*$^{AA/AA}$*/TRAMP* and *WT/TRAMP* mice, and both strains developed prostate adenocarcinomas, suggesting that IKKα kinase activity is required for CaP progression but not for normal prostate development and early tumorigenesis (Fig. 2).

We also found that the expression of some cyclins (cyclin A, cyclin B) was decreased in *Ikkα*$^{AA/AA}$*/TRAMP* tumors as compared with *WT/TRAMP* tumors (Fig. 3). A metastasis suppressor Maspin was down-regulated in wt tumors but not in *Ikkα*$^{AA/AA}$ tumors (Fig. 4), suggesting that IKKα activation might repress Maspin expression which was supported by *in vitro* Maspin-luciferase reporter experiments (Fig. 5).

2. Construct mice with a specific deletion of *Ikkβ* in prostate epithelial cells and examine whether this deletion inhibits prostate carcinogenesis in the TRAMP model, and whether IKKβ play a role in the transition from AD CaP to AI CaP

To test the role of the IKKβ subunit in CaP development, we constructed a mouse strain that contains a prostate epithelium-specific deletion of the gene coding for the IKKβ catalytic subunit. In these experiments we took advantage of the *Ikkβ*$^{F/F}$ mouse strain, , which harbors a conditional loss-of-function " floxed" *Ikkβ* allele. To delete IKKβ in prostate epithelial cells we crossed *Ikkβ*$^{F/F}$ mice to *PB-CRE4* transgenic mice, which express CRE recombinase in prostate epithelial cells. This yielded an *Ikkβ*$^{F/+}$*PB-CRE4* heterozygote strain, which after intercrossing generated a homozygote *Ikkβ*$^{F/F}$*PB-CRE4* mouse. We examined the efficiency of IKKβ deletion in the prostate and in purified prostate epithelial cells from 10-12 week old male *Ikkβ*$^{F/F}$*/PB-CRE4* mice by polymerase chain reaction (PCR) and immunoblotting and found efficient deletion of the *Ikkβ*^{F} allele and absence of IKKβ protein in purified ventral and dorsolateral prostate gland epithelial cells from *Ikkβ*$^{F/F}$*/PB-CRE4* mice (Fig. 6). No differences in the size of the prostate gland and its histological composition between *Ikkβ*$^{F/F}$ and *Ikkβ*$^{F/F}$*/PB-CRE4* mice were observed. Thus, IKKβ is not required for normal prostate development and maintenance. Since effective and prostate-specific deletion of IKKβ has been confirmed, we crossed recombinant *Ikkβ*$^{F/F}$*PB-CRE4* mice as well as *Ikkβ*$^{F/F}$ mice with the *TRAMP* transgenic mouse to generate *Ikkβ*$^{F/F}$*PB-CRE4-TRAMP* and *Ikkβ*$^{F/F}$*-TRAMP* mice. Cohorts of 15 male mice of the appropriate genotypes (*Ikkβ*$^{F/F}$*PB-CRE4-TRAMP* and *Ikkβ*$^{F/F}$*-TRAMP*) are currently being monitored for external signs of prostate cancer formation. Mice that will exhibit large palpable tumors will be sacrificed and both primary and metastatic tumor tissues will be collected for primary cell culture, histological and biochemical analyses.

To examine whether IKK/NF-κB is activated during the evolution of AI CaP, the androgen-dependent

(AD) human CaP cell line LNCaP was inoculated subcutaneously into immunocompromised SCID mice. When tumor mass reached 1 cm^3, one half of the tumor-bearing mouse cohort was sacrificed and AD tumor tissue was collected. The other half of the mouse cohort was castrated. Two months later when the tumors in the castrated mice re-grew, mice were sacrificed and tumor tissues were collected to yield AI tumors. Protein lysates derived from AD and AI tumors were assayed for NF-κB DNA binding activity by an electrophoretic mobility shift assay (EMSA) and for IKK activity by an immunocomplex kinase assay. The results clearly demonstrate that both NF-κB and IKK activities are markedly elevated in AI tumor tissues (Fig. 7).

These results support the notion that the IKK-NF-κB pathway may play an important role in development of AI CaP. It was reported that 80% of TRAMP mice castrated at 12 weeks of age will develop aggressive AI CaP at 24 weeks of age. To determine the effect of IKKβ ablation on development of androgen-independent prostate cancer (AI CaP) in TRAMP mice, cohorts of 20 male mice of each genotype (*Ikkβ$^{F/F}$/PB-CRE4-TRAMP* and *Ikkβ$^{F/F}$/TRAMP*) are being prepared and will be castrated at 12 weeks of age and sacrificed at 24 weeks of age. Size, weight and histology of the prostate tissue and of primary and metastatic CaPs will be measured and recorded. Prostate tissues, primary and metastatic CaP will be collected for primary cell culture, histological and biochemical analyses.

While we have been breeding the mice described above, we have cultured primary mouse CaP cells isolated from *Ikkβ$^{F/F}$/TRAMP* mice, which contain two "floxed" *Ikkβ* alleles that can be deleted by expression of CRE-recombinase. These cells are mixtures of tumor stroma cells and epithelial cancer cells. Cells were infected with CRE-adenovirus to delete both *Ikkβ* alleles in both cell types or infected with GFP-adenovirus as a control. IKKβ deletion efficiency was examined by Western blotting. *Ikkβ*-deleted cells and non-deleted cells were transplanted into the flank of male *Rag1$^{-/-}$* mice. Two months later mice were sacrificed and tumor weights were measured. Some of the mice were castrated one month after transplantation, and two months after castration mice were sacrificed and tumor weights were measured. We found that *Ikkβ* deletion in tumor cells decreased tumor growth after castration (Fig. 8).

The stroma and extracellular matrix are essential for functional and morphological differentiation of the prostatic epithelium. It is also postulated that the prostate stroma may plays an important role in CaP development, as we found that the ratio between the stroma and epithelial cancer cells was increased in castrated TRAMP prostate tumors (Fig. 9). We found that both IKK and NF-κB activities were increased in castrated CaP (Fig. 10), and *Ikkβ* deletion in tumor tissue cells (both stroma and epithelial cancer cells) decreased tumor growth after castration. Analysis of the role of IKKβ either in prostate epithelial cancer cells or stromal components in AI CaP development will provide us with important information as to the cell-type specificity of IKKβ function. We also separated epithelial cancer cells and tumor stromal cells from CaP insolated from *Ikkβ$^{F/F}$/TRAMP* mice. IKKβ *was* deleted by infection with CRE-adenovirus in either stromal cells or in cancer cells. Equal number of epithelial cancer cells and stromal cells of different genotypes will be mixed in different combinations (*Ikkβ$^{F/F}$* stroma+ *Ikkβ$^{-/-}$* CaP; *Ikkβ$^{-/-}$* stroma + *Ikkβ$^{F/F}$* CaP; etc). To examine the tumor development, equal numbers of cell combinations will be implanted under the renal capsule of male SCID mice (5 mice for each cell combination). One month later, mice will be castrated, two months later, mice will be sacrificed, and the size and weight of each tumor will be measured and recorded. Tumor tissues will be collected and analyzed. These experiments are ongoing.

Table 1: **Incidence of metastasis at death of WT/TRAMP and IKKαAA/TRAMP mice**

	PLA(%)	Kidney+nodes (%)	Liver (%)	Lung (%)
WT/TRAMP (n=23)	**20 (87)**	**10 (43)**	**4 (17)**	**5 (22)**
IKKαAA/TRAMP (n=22)	**9 (41)**	**4 (18)**	**0 (0)**	**0 (0)**

Fig. 1

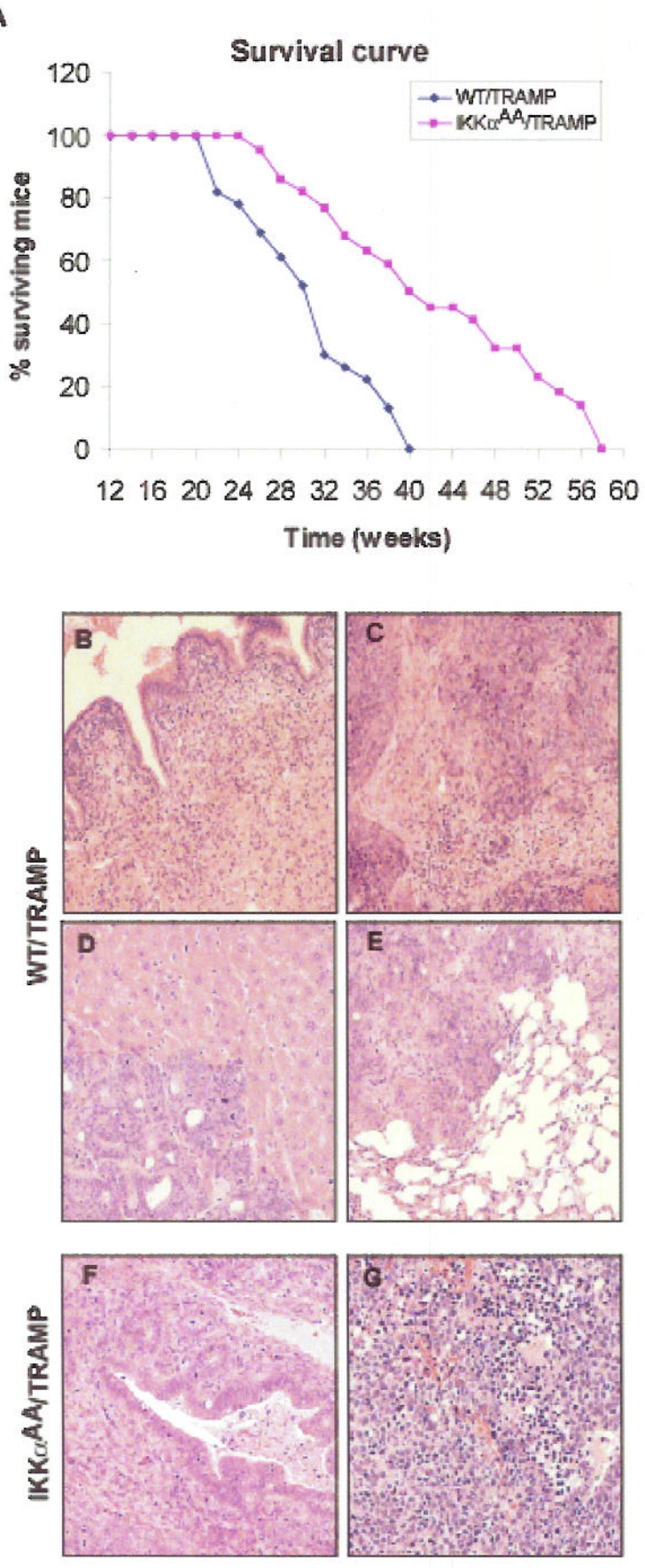
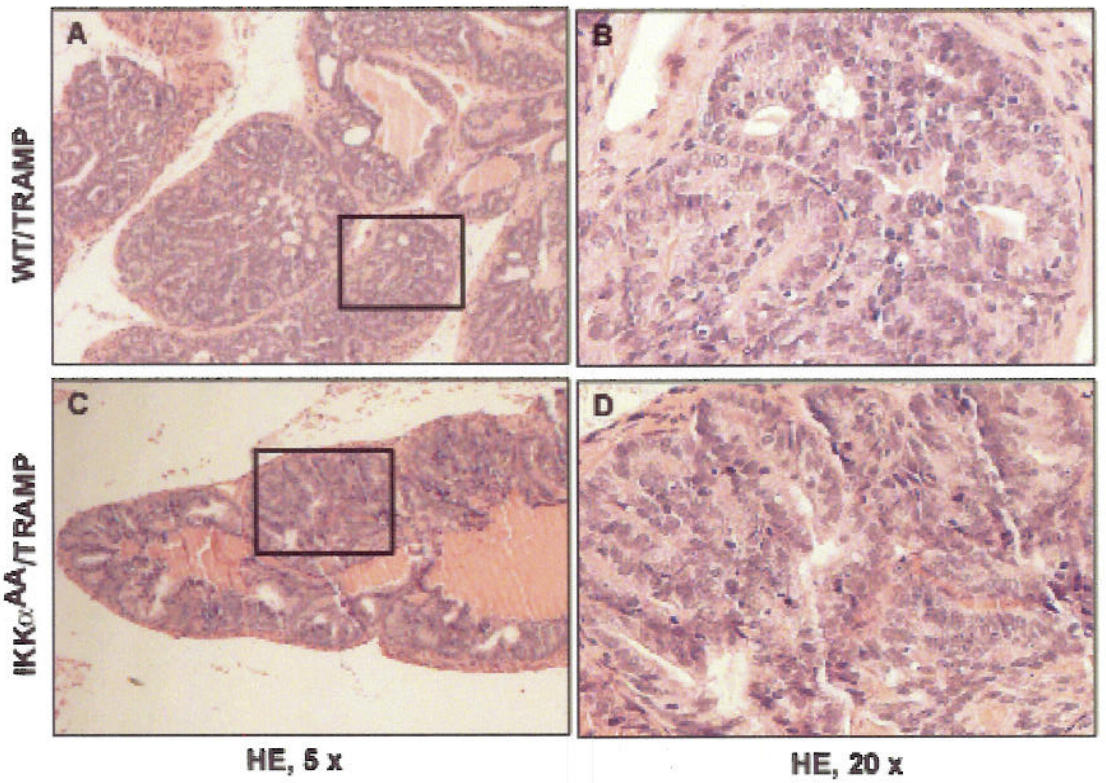
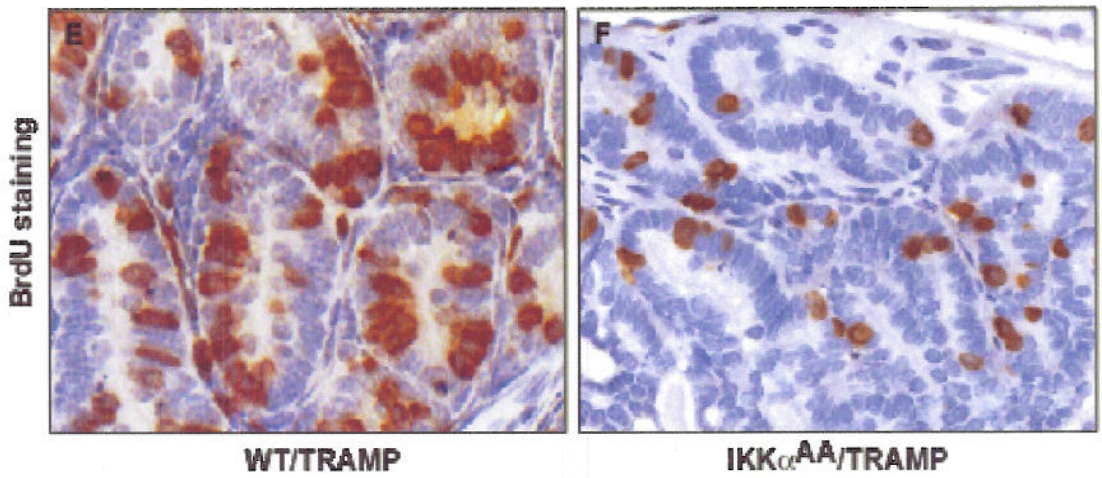

A
Survival curve
120
100
80
60
40
20
0
% surviving mice
WT/TRAMP
IKKα^AA/TRAMP
12 16 20 24 28 32 36 40 44 48 52 56 60
Time (weeks)
B
C
D
E
F
G
WT/TRAMP
IKKαAA/TRAMP

Fig. 2


A
B
C
D
WT/TRAMP
IKKα^AA/TRAMP
HE, 5 x
HE, 20 x


E
F
BrdU staining
WT/TRAMP
IKKα^AA/TRAMP

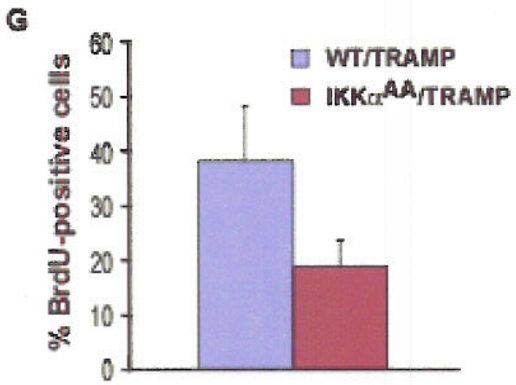
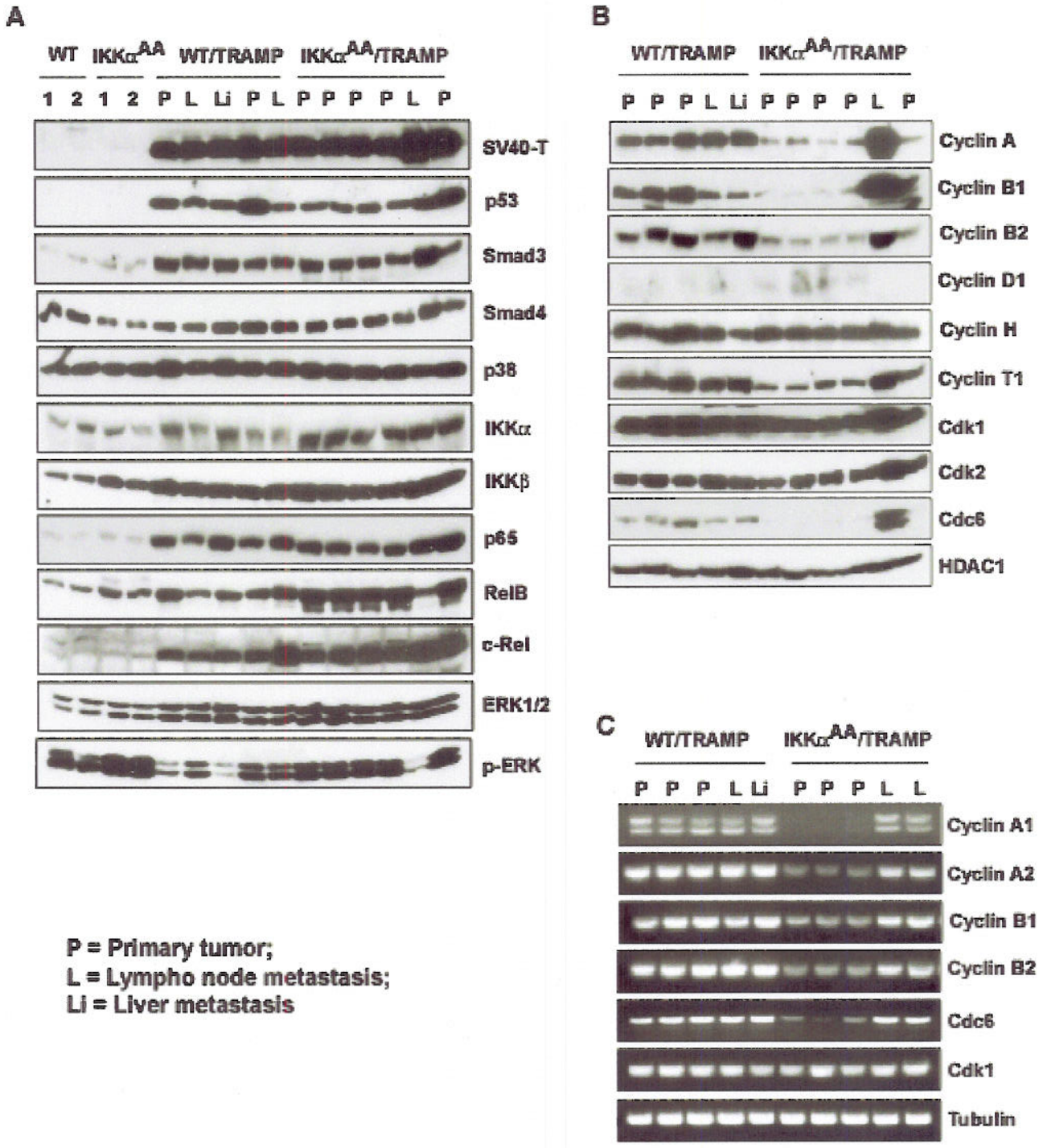

G
% BrdU-positive cells
0
10
20
30
40
50
60
WT/TRAMP
IKKα^AA/TRAMP

Fig. 3



P = Primary tumor;
L = Lympho node metastasis;
Li = Liver metastasis

Fig. 4

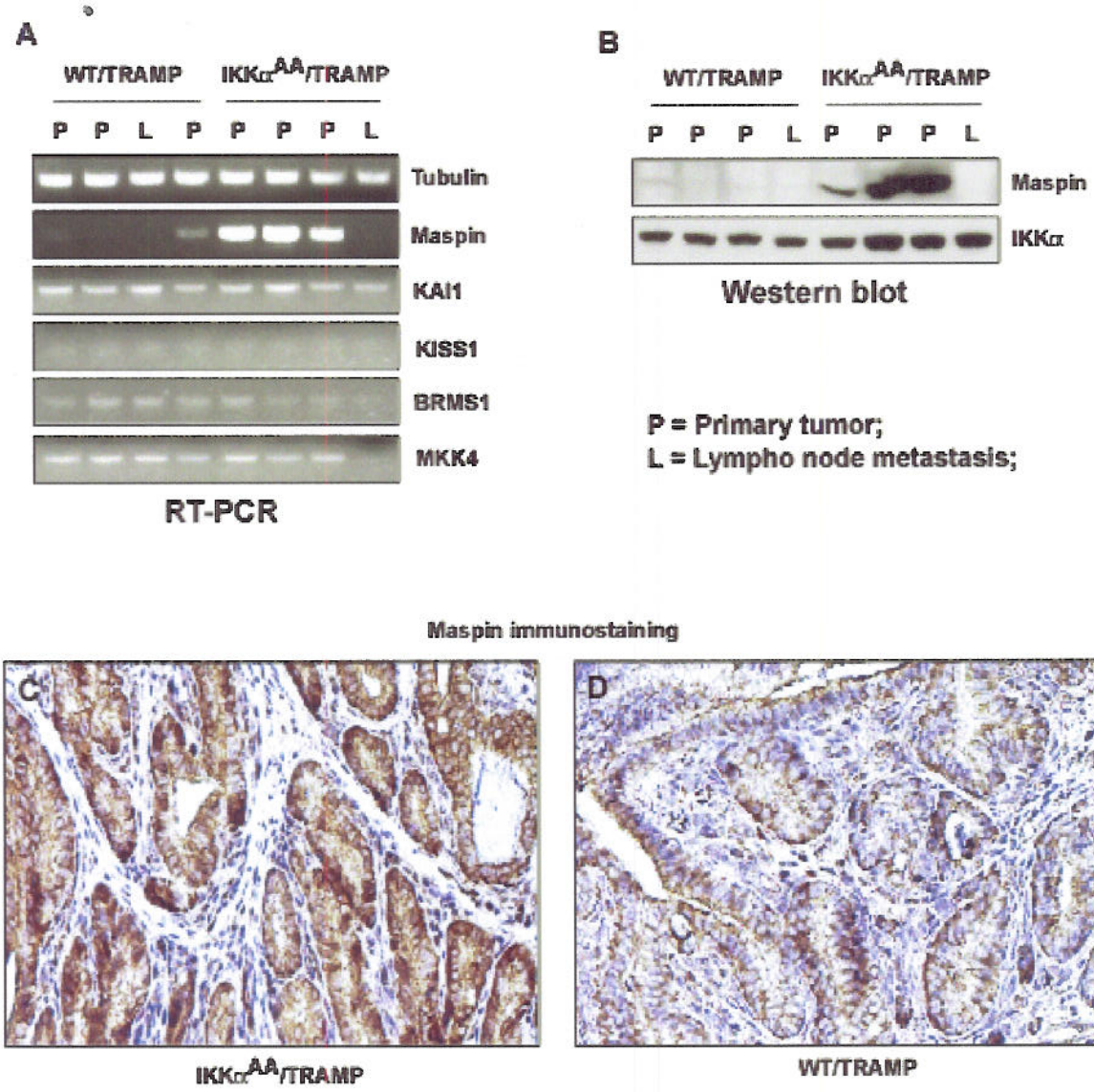
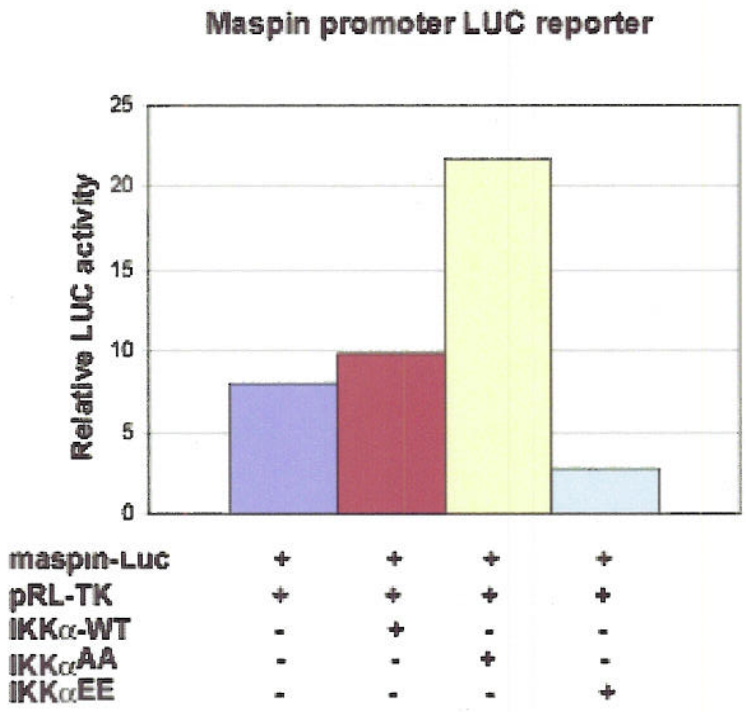

A
WT/TRAMP
IKKα^AA/TRAMP
P P L P P P P L
Tubulin
Maspin
KAI1
KISS1
BRMS1
MKK4
RT-PCR
B
WT/TRAMP
IKKα^AA/TRAMP
P P P L P P P L
Maspin
IKKα
Western blot
P = Primary tumor;
L = Lympho node metastasis;
Maspin immunostaining
C
IKKα^AA/TRAMP
D
WT/TRAMP

Fig. 5


Maspin promoter LUC reporter
Relative LUC activity
25
20
15
10
5
0
maspin-Luc + + + +
pRL-TK + + + +
IKKα-WT - + - -
IKKαAA - - + -
IKKαEE - - - +

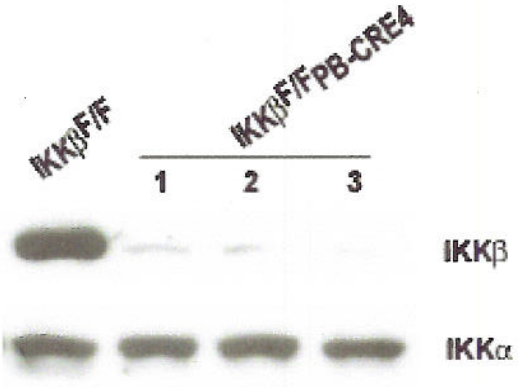


Fig 6, IKKβ protein is efficiently deleted in prostate epithelial cells in IKKβ$^{F/F}$PB-CRE4 mice. Purified ventral and dorsolateral prostate epithelial cells from 10-12 week old male IKKβ$^{F/F}$ and I KKβ$^{F/F}$PB-CRE4 mice were lysed for immunoblot analysis. The results show efficient deletion of IKKβ in ventral and dorsolateral prostate epithelial cells from IKKβ$^{F/F}$PB-CRE4 mice .

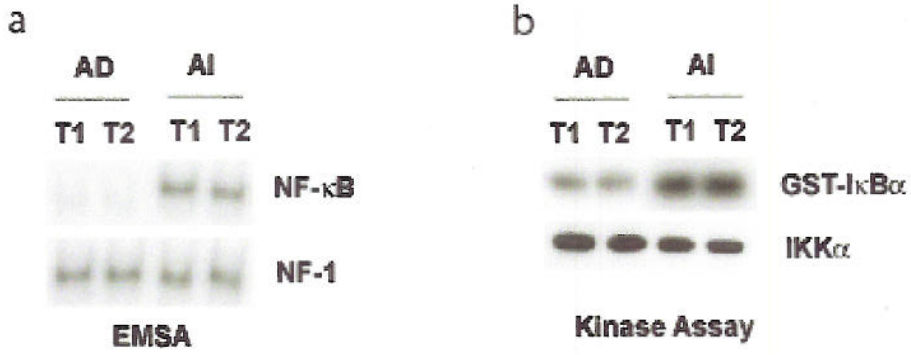
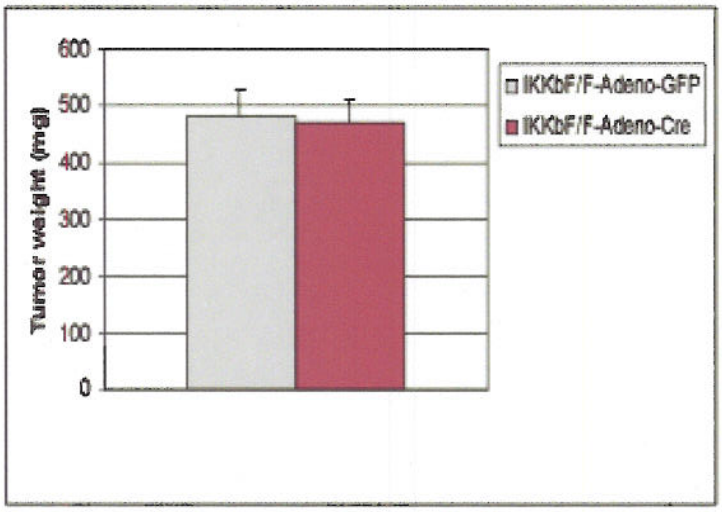


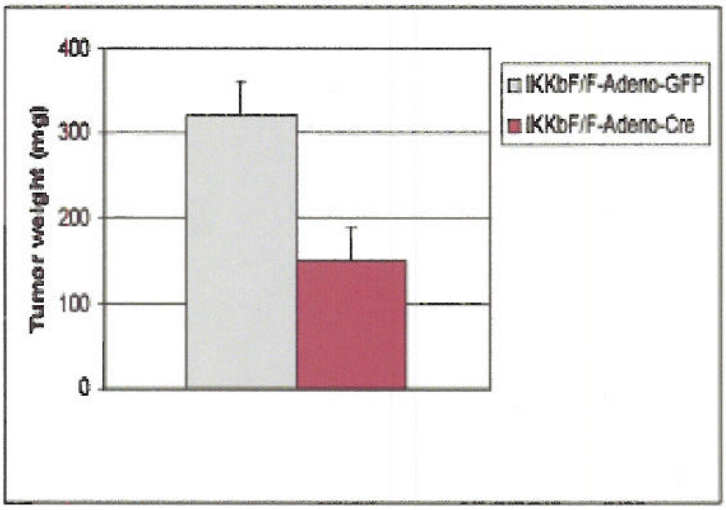
Fig 7, Human LNCaP cells were implanted subsutaneously into SCID mice. When the tumor mass reached 1 cm^3, half of the tumor-bearing mice were sacrificed and tumor tissues were collected (androgen-dependent tumors, AD), The remaining half were castrated. Two months later when the tumors in the castrated mice regrew, the mice were sacrificed and tumors were collected (androgen-independent tumors, AI). Tumor extracts were used for electrophoretic mobility shift assay (EMSA) (a) and determining of IKK kinase activity (b). Both NF-κB DNA binding activity and IKK kinase activity were markedly increased in AI tumors as compared to AD tumors.

Figure 8

Deletion of IKKß in primary mouse tumor tissue cells inhibits tumor growth when host Rag1-/- mice are castrated



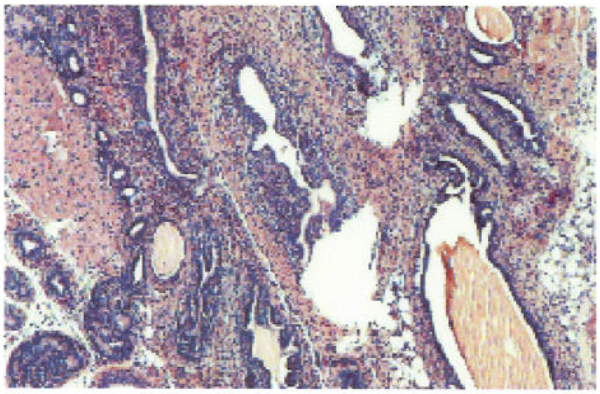
Primary tumor tissue cells were inoculated in the flank of Rag1-/- mice, two months later mice were sacrificed and tumor weight was measured



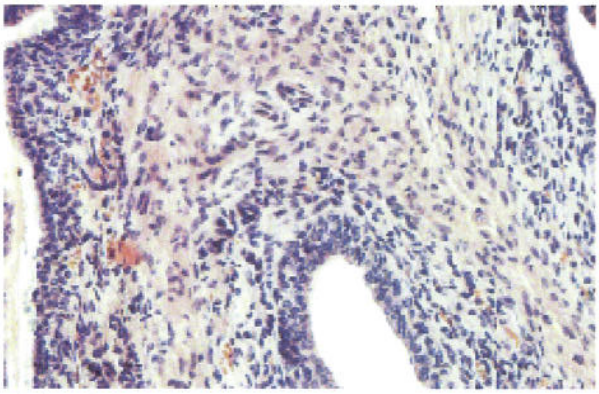
Primary tumor tissue cells were inoculated in the flank of Rag1-/- mice, one month later mice were castrated. After two months of castration mice were sacrificed and tumor weight was measured

Figure 9

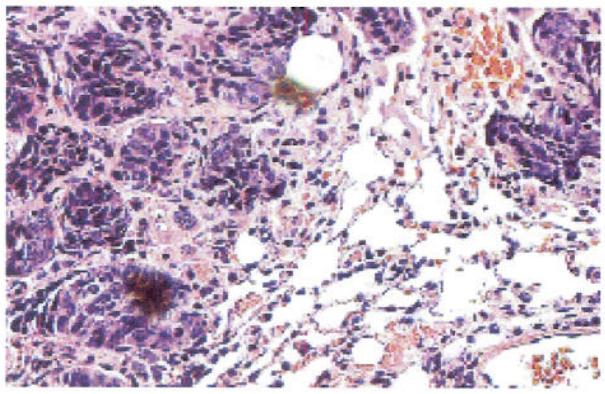
Pathology of prostate adenocarcinoma in castrated TRAMP mice



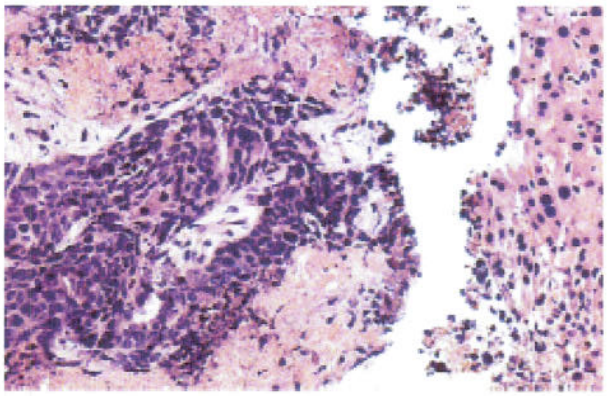
Primary prostate adenocarcinoma, 5x



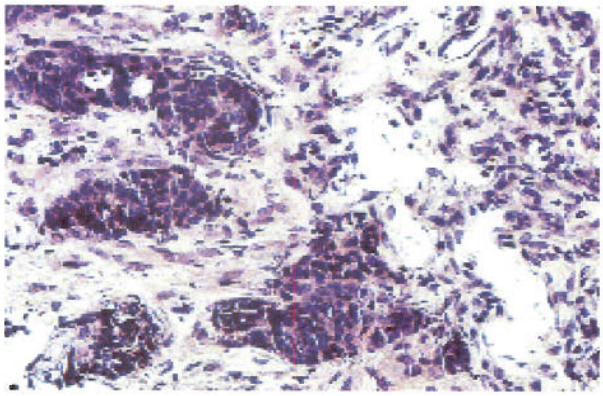
Primary prostate adenocarcinoma, 20x



prostate adenocarcinoma, lung metastasis 20x



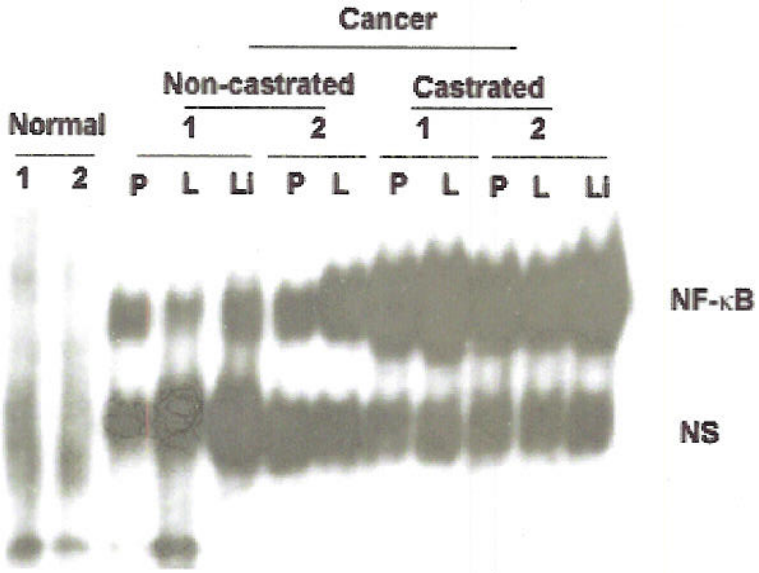
prostate adenocarcinoma, liver metastasis 20x



prostate adenocarcinoma, lymph node metastasis 20x

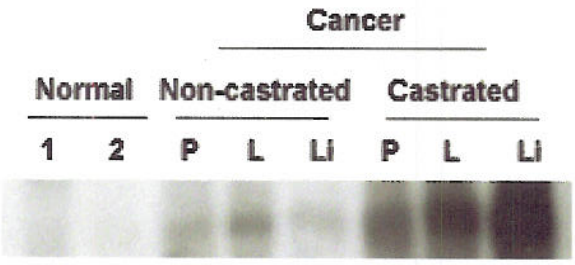
Figure 10

A



EMSA using tissue lysates from normal mouse prostate or mouse prostate cancer
P = Primary tumor;
L = Lympho node metastasis;
Li = Liver metastasis

B



IKK Kinase Assay using tissue lysates from normal mouse prostate or mouse prostate cancer
P = Primary tumor;
L = Lympho node metastasis;
Li = Liver metastasis

Conclusions

In summary, results obtained during the past project year suggest that both IKKα and IKKβ may play an important role in development and progression of CaP. Clearly, a more thorough examination of the role of the two IKK subunits in CaP development and progression is needed, especially during the transition of CaP from an AD to an AI state. These are functions being examined during the current project year. Most interesting is the finding of maspin upregulation in CaP from *Ikkα$^{aa/aa}$/TRAMP* mice. If maspin will be verified to be a tumor and metastases suppressor in this model as well as in human CaP, our work provides a clear outline for the development of new therapeutic approach to prostrate cancer.

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